Genomic Response to Growth Factors: Daniel Nathans, Senior Investigator
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As noted in the Annual Report, my laboratory has been interested in defining a genetic program induced in cultured murine 3T3 cells by serum growth factors. Our overall objective is to contribute to understanding how polypeptide growth factors stimulate cell proliferation, a question with general relevance to cellular responses to extracellular signalling agents during development and in the adult animal. To define growth factor-induced genes, some years ago we isolated from BALB/c 3T3 cells cDNAs derived from mRNAs that appeared at various times after non-growing cells were stimulated with serum. Serum causes essentially all the cells to enter the cell cycle and undergo synchronous DNA replication after 12 to 15 hours followed by mitosis and cell division. Our initial results indicated that specific mRNAs appear sequentially after serum stimulation. Within minutes after addition of serum, a specific set of genes is transcriptionally activated, including proto-oncogenes fos and myc (as discovered by others). Since these genes are activated by serum, PDGF or FGF even in the presence of an inhibitor of protein synthesis, they comprise the primary genomic response to serum factors and have been called "immediate early" or "competence" or "early response" genes. Other mRNAs appear later and their appearance is blocked by protein synthesis inhibitors. We call RNAs of this type that appear prior to DNA synthesis "delayed early" mRNAs. Our research over the past three years concerns the characterization of immediate early genes and their encoded proteins and initial characterization of cDNAs corresponding to delayed early genes.

Immediate early genes and proteins

Lester Lau, a post-doctoral fellow, isolated a set of cDNAs from mRNAs that are superinduced when confluent, non-growing 3T3 cells maintained in 0.5% serum-containing medium are exposed to high concentrations of serum or PDGF in the presence of cycloheximide (1,2). All the corresponding genes are transiently activated as determined by nuclear run-on assays, and in most cases their mRNAs are labile (half-life of 15 to 20 minutues) (2). Of the ten cDNAs analysed (the sequences of half of these were completed in Dr. Lau's laboratory at Northwestern University), six encoded proteins whose sequences provided clues to function: three are related to known transcription factors, another has a cysteine/histidine repeat suggestive of a novel zinc finger structure, one is a transmembrane protein related to human tissue factor, and the sixth has a hydrophobic N-terminal sequence suggestive of a secreted protein.

Transcription factors

Jun family. The first of our cDNA clones sequenced encoded a protein with a cluster of positively charged amino acid residues similar to that found in Fos. We soon noticed that the protein was closely related to the sequence of v-Jun (just then reported by Peter Vogt and his colleagues), later shown to be related to the transcription factor AP-1. Our isolate, which we call jun-B, was different from the proto-oncogene c-jun based on a comparison of mouse, human, and chicken genomic Southern blots using v-jun or jun-B probes (3). jun-B cDNA was used as a probe to isolate two related cDNAs from the immediate early library. One of these was the murine c-jun and the other a new member of the jun family, called jun-D. The three murine Jun proteins are closely related to one another, particularly in their C-terminal DNA-binding

domains, i.e., the leucine repeat and the adjacent basic region, and in acidic regions nearer the N-terminus, whereas other regions of the proteins are divergent. Like jun-B, c-jun is also a typical immediate early gene in its response to serum or PDGF, whereas jun-D is expressed in resting cells and shows only a slight increase in expression after addition of serum (4,5). In 3T3 cells we found that jun-B mRNA increased markedly in response to forskolin and phorbol esters, calcium ionophore, and high K^+ concentration, and in macrophages, to interferons α, β, or γ, whereas c-jun and jun-D mRNAs showed little change under these conditions. In mouse tissues and cell lines the three jun genes are widely expressed, but the ratios of their mRNAs vary greatly. These various differences in expression are reflected in the marked differences we found in the nucleotide sequences upstream of each gene. We do not yet know what upstream sequences are important for the growth factor response of jun-B and c-jun.

Our experiments on the DNA-binding properties of the Jun proteins alone or as Fos or Fra-1 heterodimers (6) are summarized in the accompanying 1988-'89 Annual Report, as are those comparing the specificity for the DNA-binding sites of Jun/Fos chimeric proteins with either Jun or Fos basic regions (7). (These experiments were carried out by Yusaku Nakabeppu.)

Recently we have prepared c-Jun and Fra-1 in E. coli. These proteins show little binding to an AP-1 site unless they are first incubated with an S-100 mammalian cell extract. (We are tracking down the basis of this effect.) Of interest is the fact that both "activated" c-Jun alone and "activated" Fra-1 alone bind to an AP-1 site. In preliminary experiments the activated proteins stimulated in vitro transcription of DNA with an AP-1 responsive promoter, Jun + Fra-1 being more active than the sum of each alone.

Zinc finger proteins. Barbara Christy found that one of the immediate early DNA clones (zif/268) encodes a 431 amino acid protein with three zinc fingers of the TFIIIA type (8). (The same or homologous cDNA has been isolated in three other laboratories.) Zif268 is localized to the nucleus and has a short half-life. Its mRNA is most abundant in brain, lung, and heart. The promoter region of the gene has a number of potential binding sites for known transcription factors including four potential serum response elements (SREs) similar to that described in the fos promoter region and shown to confer serum responsiveness to other genes. Christy showed that all four zif268 SRE-like sequences bind serum response factor (the protein that binds to the fos SRE) and confer serum responsiveness (9). These results suggest that the SREs may be responsible for co-induction of fos and zif268. Recently, using Zif268 prepared in E. coli, we identified a strong DNA-binding site for Zif268, namely the sequence GCG$\begin{matrix}\mathrm{G}\\\mathrm{T}\end{matrix}$GGGCG (9). One or more Zif268 sites are upstream of the immediate early genes zif268, nup475, mur77, and jun-B, suggesting that Zif268 may regulate these genes. In preliminary experiments Christy has found in transfection assays that transcription from the zif268 promoter is down-regulated by co-transfection with a plasmid that expresses Zif268.

Nur77 is another immediate early zinc finger protein identified via the sequence of its cDNA. (Similar cDNAs have been isolated in two other laboratories.) Nur77 is a new member of the glucocorticoid-thyroid hormone receptor superfamily. Its ligand is unknown. The Nur77 protein and gene are being studied in Lester Lau's laboratory.

A third possible zinc finger protein is encoded by cDNA clone 475, whose sequence was determined by Raymond DuBois. By immunofluorescence the predicted protein is nuclear (we call it Nup475), and is rich in glycine, serine, threonine and proline. It is not related to sequences in the DNA or protein data banks. Nup475 has two adjacent cysteine/histidine-containing sequences of the form $CX_8CX_5CX_3H$, and the protein produced in E. coli binds zinc. We speculate that the cysteine/histidine sequences may form a novel zinc finger structure that binds to a specific DNA sequence, and that Nup475 is another immediate early transcription factor. We are now trying to determine whether Nup475 binds to a specific DNA sequence.

Transmembrane protein. One of the immediate early cDNAs (clone 482) encodes a transmembrane protein (called mTF) analysed by Stephen Hartzell and Anthony Lanahan (11) that is the murine homolog of human tissue factor, a membrane protein involved in initiating blood clotting after tissue injury. Hartzell showed that mTF produced in vitro is inserted into microsomal vesicles and is N-glycosylated, probably at each of the four sites predicted from the sequence. It is also on the surface of proliferating 3T3 cells. We don't know what role mTF may play (if any) in the growth response. For the time being we have put mTF aside.

Delayed early genes and proteins

Our first cDNA isolates included one that encoded proliferin (PLF), a new member of the prolactin-growth hormone family. In our last work on proliferin Se-Jin Lee found that the form secreted by cultured cells contained mannose-6- phosphate and bound to the high molecular weight mannose-6-phosphate receptor (12). In the course of those experiments Lee discovered that the level of active mannose-6-phosphate receptor was very high in mouse fetal liver membranes (and membranes from pregnant mice) and abruptly decreased post-natally. Lee also localized proliferin and its mRNA to the same giant trophoblastic cells of the mouse placenta that secrete placental lactogens (13). Daniel Linzer, who discovered proliferin while in my laboratory, is continuing to study it at Northwestern University.

More recently Anthony Lanahan has isolated from a 3T3 cell cDNA library a number of cDNA clones that are derived from delayed early mRNAs. These RNAs appear three to five hours after serum stimulation of non-growing cells and reach maximal levels well before the onset of DNA synthesis. Their appearance is blocked by cycloheximide, and therefore may be dependent on immediate early transcription factors.

Related Genomic Response in Neurons

David Saffen, while a post-doctoral fellow in my laboratory, initiated a series of collaborative experiments to determine whether the immediate early genes induced in 3T3 cells by serum growth factors were also induced in brain by depolarizing stimuli (14). This followed a report that fos mRNA is induced in rat brain after administration of a convulsant. Saffen, in collaboration with A. Cole, P. Worley and J. Baraban (of the Neuroscience Department at Hopkins) found that c-jun, jun-B, and zif268 in addition to fos mRNA increased markedly in the hippocampus and dentate gyrus of rats given convulsant. The time course was very similar to that seen for these RNAs in serum-stimulated 3T3 cells. It thus appears that some of the same transcription factors are involved in the immediate early gene response of brain neurons, in 3T3 cells, and in hepatocytes after partial hepatectomy (15) (as well as NGF-stimulated PC12 cells studied by others). These observations on the genomic response of neurons to depolarizing signals are being followed up by Worley and Baraban.

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7. Y. Nakabeppu and D. Nathans. The Basic Region of Fos Mediates Specific DNA Binding. EMBO J. 8:3833-3841, 1989.
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12. S-J. Lee and D. Nathans. Proliferin Secreted by Cultured Cells Binds to Mannose 6-Phosphate Receptors. J. Biol. Chem. 263:3521-3527, 1988.
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Future Research Plans

I intend to continue to explore in cultured cells several facets of the growth factor-induced genetic program and its role in regulating cell proliferation. We will concentrate on the immediate early transcription factors we have identified, their potential target genes, and the proteins encoded by delayed early genes in the expectation that one or more of these proteins may participate in the regulation of DNA synthesis. I expect some of my Associates to continue their projects after they leave my laboratory.

Activation of transcription factor genes. Immediate early genes are activated during the first few minutes following serum or growth factor stimulation of 3T3 cells, even in the presence of an inhibitor of protein synthesis. The hypothesis we will explore (based largely on studies of NFκB and gene induction by interferon) is that pre-existing, inactive transcription factors(s) are modified or otherwise activated immediately after growth factor-receptor interaction and that the active factor(s) bind to specific site(s) upstream of one or more immediate early genes, thereby activating transcription. We will concentrate on jun-B and nup475. The first series of experiments will be aimed at identifying in transfected plasmids cis elements needed for activation. Our initial experiments suggest that there may be negative elements involved in repression of jun-B and nup475 in quiescent cells, i.e., the jun-B or nup475 promoters linked to CAT are nearly as active in serum-deprived 3T3 cells as in stimulated cells (in contrast to zif268 and fos constructs). We will try to localize these elements as well as PDGF-responsive, positive regulatory elements using the respective genes together with 5' and 3' flanking sequences. If novel cis elements are localized we will identify element-binding proteins, purify them, clone their cDNAs, and study their roles in regulating the genes. As mentioned, I expect some of the work growing out of these experiments to be continued by my Associates after they leave my laboratory.

Target genes. Our hypothesis is that immediate early transcription factors down-regulate immediate early genes and activate delayed early genes that make up the growth factor-induced genetic program. We are now exploring the possibility that Zif268 down-regulates its own gene and some of the other immediate early genes containing Zif268 binding sites in their promoters. As mentioned, we have indications of negative autoregulation by Zif268 in transfection experiments, but we don't yet know if this is a direct effect. The simplest possibility is that binding of Zif268 to its sites upstream of the zif268 gene is an essential step. Experiments to test the effect of changes in binding sites and the Zif268 protein sequence will be carried out to clarify this and to determine what parts of the protein are important. We also plan to do parallel in vitro transcription experiments with purified Zif268 produced in E. coli.

To look for delayed early gene targets of the transcription factors we will use the cDNAs recently isolated in my laboratory by Lanahan. In one series of experiments we plan to use cell lines expressing Jun-B, Fos, or Zif268 fused to the estrogen-binding domain of the estrogen receptor; analogous fusion proteins have been shown by others to be inactive until activated by estrogen. If the DNA-binding activities of the fusion proteins can be activated by estrogen, we will look for the induction of transcription of delayed early genes. In a more direct approach we will analyse the promoter regions of the delayed early genes and look for AP-1, Zif268 (and perhaps Nup475) binding sites. The regulation will be analysed in detail by appropriate transfection and

mutagenesis studies.

Activities of transcription factors. Our main focus will be to compare the in vitro transcriptional activities of Jun proteins and various Jun/Jun and Jun/Fos heterodimers to deterimine if there are effects specific to a given dimer. As mentioned, we have prepared c-Jun and Fra-1 in E. coli. So far we have been unable to prepare full length Jun-B, Jun-D, Fos, and FosB in E. coli. Therefore we have turned to cos cells transfected with amplified plasmids that express each of the members of the Jun and Fos families. Our plan is to purify each of these proteins using as a final step specific antibody columns (four of the antisera are in hand) and to test the various dimer combinations for transcriptional activity on promoters with binding sites, including (we anticipate) those from delayed early genes. Once the in vitro experiments are progressing, we expect to localize protein domains essential for the transcriptional effects. Later we will try to identify transcription components that interact with Jun/Fos dimers. (As indicated earlier, my former post-doctoral fellow Kevin Ryder is continuing related experiments on Jun/Fos transcriptional activities in transfected cells.)

Our experiments with Jun/Fos chimeric proteins described earlier have led us to search for potential Jun/X and Fos/X heterodimers that may bind to novel, assymmetrical DNA binding sites, in accordance with suggestion for transcription factors with leucine zippers. Purified ^{125}I-c-Jun and ^{125}I-Fra-1 (or binding domains) will be used to screen a λgt11 mouse expression library for proteins that bind the iodinated probe. Positive cDNAs and their encoded proteins will be analysed for DNA binding properties as homodimers and heterodimers. We are now testing the sensitivity of the screening assay using E. coli infected with λ recombinants encoding c-Jun or Fra-1.

Delayed early proteins. We are just collecting sequence data on our cDNA isolates derived from delayed early mRNAs. I will be especially interested in those encoding novel nuclear proteins, or protein-modifying enzymes as potential regulators of DNA replication. We hope to get clues from the sequences of the proteins and their cellular localization, as we did with the immediate early proteins. I am also interested in a comparison of delayed early proteins in different cell types that have similar immediate early transcription factor responses to signalling agents. To this end we are collaborating with Paul Worley and Jay Baraban in the isolation and analysis of delayed early cDNAs from rat hippocampus following electrical stimulation. The nature of the encoded proteins may also provide clues to long term neuronal effects of depolarizing stimuli.

Genes activated during the cell cycle. Using RNA prepared from proliferating mouse L cells fractionated by centrifugal elutriation, we have found that immediate early RNA levels show little variation over the cell cycle. Ruti Parvari has therefore begun to screen G1-, S-, and G2-enriched cDNA libraries for growth phase-specific mRNAs. We will also determine whether any of our delayed early RNAs fluctuate during the cell cycle. Our objective is to identify genes (other than histone and dihydrofolate reductase genes) that are activated at specific points in the mammalian cell cycle and study their encoded proteins in relation to regulation of the cell cycle.

Professional personnel

		Appointment dates
Barbara A. Christy	HHMI Associate	10/16/86
Anthony A. Lanahan, Jr.	" "	06/01/87
Raymond N. DuBois	" "	07/01/88
Yusaku Nakabeppu	" "	03/01/89
Mary Beth Murray	" "	10/01/89
Ruti Parvari	Chaim Weizmann Fellow	
(supported by Chaim Weizmann Award)		
Evelio Perez-Albuerne	MD/Phd student	
(supported by Medical Scientist Training Program, NIH)		
Pierre-Yves Chevray	MD/PhD student	
(supported by Medical Scientist Training Program, NIH)		

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Reprints attached (work done in past three years)

1. K. Ryder, L. F. Lau, and D. Nathans. A Gene Activated by Growth Factors is Related to the Oncogene v-jun. Proc. Natl. Acad. Sci. USA 85:1487-1491, 1988.

2. Y. Nakabeppu, K. Ryder, and D. Nathans. DNA-binding Activities of Three Murine Jun Proteins: Stimulation by Fos. Cell 55:907-915, 1988.

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